Characterization of a cyclodextrin-oligonucleotide complex by capillary electrophoresis using laser-induced fluorescence

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Dedicated to the memory of Prof. Antoine (Tony) A. Noujaim, in recognition of his outstanding contributions to radiopharmacy, diagnostic oncology and the immunotherapy of cancer

Received November 29, 2006; Revision received March 22, 2007; Accepted March 26, 2007, Published March 29, 2007.

ABSTRACT - PURPOSE: Cyclodextrins (CDs) have been identified as a viable alternative to viral vectors for use in therapeutic applications. Here, the stability of the complex formed between the a multiply-charged, cationic, fully-substituted heptakis-(6-amino-2-galactosyl)cyclodextrin (BCD-X12) with a multiply-charged 12-mer hexachlorofluorescein-tagged arabinopolynucleotide (Hex-PAH) have been evaluated. **METHODS:** The stability of complexes of Hex-PAH and BCD-X12 was studied with respect to mole ratio (1:1, 1:2, and 1:5 Hex-PAH:BCD-X12), pH, buffer concentration, temperature, and agitation using capillary electrophoresis with laser-induced fluorescence detection (CE/LIF). Two neutral CDs and an additional cationic CD were also tested under the same analytical conditions to determine their ability to form complexes. **RESULTS:** Hex-PAH:BCD-X12 complexes at mole ratios of 1:2 were stable in 10 mM (160 mM total borate concentration) sodium tetraborate buffer at pH 7.5 and at temperatures of 4 °C and 25 °C over 48 hours. However, the Hex-PAH:BCD-X12 complex was less stable at 37 °C and at higher buffer concentrations and pH values. Strong vortex mixing prior to analysis was found to disrupt the complex. Of the four CDs tested for their ability to complex with Hex-PAH, only BCD-X12 formed stable complexes with Hex-PAH under the test conditions. **CONCLUSIONS:** Capillary electrophoresis was found to be well suited to test the stability of cyclodextrin-nucleotide complexes. CE/LIF indicated that only a single Hex-PAH:BCD-X12 complex was formed at all formulation ratios, and that the complexes were electrophoretically identical to each other, and increasing the molar ratio beyond 1:2 did not contribute measurably to complex stability. Storage temperature and agitation conditions were found to influence complex stability. Since no stable complexes were formed with neutral cyclodextrins, the results support the hypothesis of a 'charge associated' complex rather than an inclusion complex, although inclusion complexes cannot be excluded on the basis of these studies.

INTRODUCTION

Recent concerns with the safety of viral drug delivery systems for nucleic acid based therapeutics has sparked research into viable non-viral delivery systems. While several delivery systems based on lipids, peptides, polymers, and dendrimers have been studied, they have had limited success. Cyclodextrins (CDs), which are cyclic oligosaccharide polymers containing 6, 7 or 8 (1, 4)-glucosidic links, denoted α–, β–, and γ–CD, respectively, offer a number of advantages for development as vectors for the delivery of nucleic acids.

Naturally occurring CDs and many of their derivatives are well researched and are known to have low toxicity and lack immunogenicity in humans (1). Their hydrophobic, cone-shaped cavities are available to form inclusion complexes with small, lipophilic compounds, and their hydrophilic shells, which contain a large number of primary (narrow side) and secondary (wide side) hydroxyls, are available for chemical modification to accommodate biochemically-functional elements. For example, cationic CD derivatives have been shown to bind more strongly to nucleotides than

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non-ionic forms due to the electrostatic interaction between the ammonium group of the hosts and the phosphate groups of the guests (2). In addition, CDs are able to protect drugs from degradation, increase the solubility of poorly soluble drugs, and enhance membrane permeability. Several groups have studied various CD architectures such as polycationic polymeric vectors and dendrimer vectors to improve drug delivery (3-8).

Capillary electrophoresis (CE) has many advantages over other separation techniques. CE requires only nanolitre volumes, has short analysis times, and when coupled with laser-induced fluorescence (LIF), has zeptomole (10^{-21}) detection limits. CE has been used extensively for the determination of binding and dissociation constants of molecular interactions including, but not limited to, peptide-drug (9), protein-ligand (10, 11), protein-DNA (12-14), protein-protein (15, 16), antibody-antigen (17), and CD inclusion complexes (18-32). Much of the work to determine the binding constants of CD inclusion complexes has been done using chiral separation (18-24); however, others have reported binding constant determination via mobility shift assays (25-32).

The CDs reported in this work have been modified to contain either or both galactose and amino moieties, fully substituted on the respective secondary or primary hydroxyl sites. Complex formation between these positively-charged CD derivatives and a 12-mer hexachlorofluorescein-tagged arabinopolynucleotide (Hex-PAH) has been investigated using CE/LIF. The objective of this preliminary work was to study the stability of the complex formed between the multiply-charged, cationic, fully-substituted heptakis-(6-amino-2-galactosyl)cyclodextrin (BCD-X12) with a multiply-charged anionic fluorescent 12-mer arabinopolynucleotide. CE/LIF was used to monitor the stability of the complexes at different borate buffer concentrations, pH values, temperatures, mole ratios, and agitation conditions.

MATERIALS AND METHODS

Reagents and Materials

The 12-mer hexachlorofluorescein-tagged arabinopolynucleotide (Hex-PAH; MW: 4616, Abs max: 535 nm, Emission max: 556 nm), heptakis-(6-amino-2-galactosyl)-β-cyclodextrin heptaacetate salt (BCD-X12, MW: 2842), heptakis-(6-azido-2-galactosyl)-β-cyclodextrin (BCD-X10, MW: 3024), and heptakis-(6-amino)-β-cyclodextrin heptaacetate salt (BCD-X4, MW: 1548), were provided by BioCyDex, Inc (Edmonton, Canada.). The structures of the BCD-X4, BCD-X10 and BCD-X12 are shown in Figure 1. β-cyclodextrin (BCD) was purchased from Aldrich Chemicals Co. (St. Louis, MO, USA). Boric acid was obtained from Fisher Scientific (Fair Lawn, NJ, USA), and sodium tetraborate was obtained from Caledon Laboratories (Georgetown, ON, Canada). Ultrapure water was obtained from a Milli-Q gradient A10 water purification system (Millipore, USA). All other chemicals were analytical grade.

Instrumentation

The laboratory-built capillary electrophoresis instrument with laser induced fluorescence (CE/LIF) detection used in this work has been previously described (14, 33). The

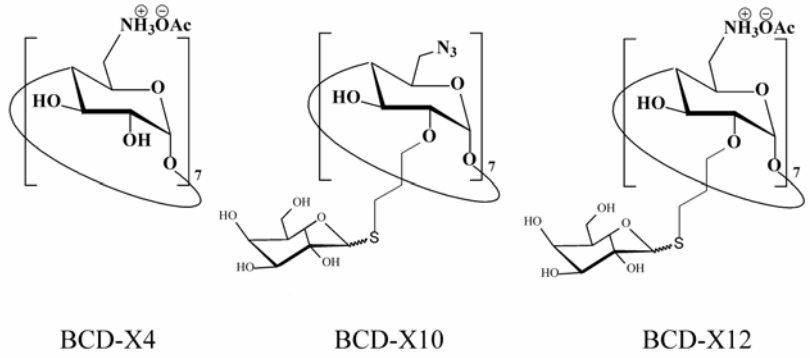

Figure 1: Structures of BCD-X4, BCD-X10, and BCD-X12.

system consists of a high-voltage CE power supply (model CZE 100R, Spellman, Plainview, NY, USA) and a fused-silica capillary (20 μm i.d., 150 μm o.d., Polymicro Technologies, Phoenix, AZ, USA), which was cut to 30 cm and inserted into a sheath flow cuvette (NSG Precision Cells, Farmingdale, NY, USA). The sheath flow solution, which was identical to the running buffer, passed the outlet end of the capillary under the conditions of laminar flow so that the solution remained constrained in a core region near the detection site. Plane-polarized light from a HeNe laser (model 05-LGP-293, Melles Griot, Carlsbad, CA, USA) with an excitation wavelength of 542.5 nm was focused using a 10 × microscope objective to a detection window near the tip of the capillary. The sample solution containing a mixture of Hex-PAH and the Hex-PAH:BCD-X12 complex was introduced into the capillary by electrokinetic injection for 5 s, and passed through the capillary at a running voltage of 667 V/cm. The fluorescence was collected at 90^{o} from the direction of excitation by a 60 × (N.A. 0.7) microscope objective (LWD-M Plan, Universe Kogaku, Japan). The transmitted light was spectrally filtered with a 560DF40 band-pass filter and detected by a photomultiplier tube (R1477, Hamamatsu Photonics, Japan). The operation of the power supply and the acquisition of data were controlled by a Power Macintosh computer with the application software written in LabView (National Instruments, Austin, TX). The capillary was washed after each run for 5 min with 20 mM NaOH followed by ultrapure water for 3 minutes, and the running buffer for 6 minutes. All experiments were done at room temperature, 22^{o}C.

Preparation of complexes

Hex-PAH was added to aqueous solutions of BCD-X12, BCD-X4, BCD-X10, or BCD in 20 mL screw-capped vials. After 2 min of vortex mixing and 5 min of sonication in a water bath, samples were shaken at 25 °C for 3 days. The equilibrated solutions were filtered through a 0.22 um filter, lyophilized (Freeze Dryer 3, Labconco, Repp Freeze Dryer, Kansas City, USA) and stored as a powder at -20 °C until use. A Hex-PAH-only solution was also prepared in the same manner. The Hex-PAH:BCD-X12 complex was prepared as described above, in three Hex-PAH:BCD-X12 mole ratios: 1:1, 1:2, and 1:5. These mole ratios represent mixing conditions only, and do not necessarily represent the actual stoichiometry of the complex. BCD-X4, BCD-X10 and BCD complexes were prepared in 1:2 Hex-PAH:CD mole ratios. Stock solutions of CD complexes and Hex-PAH were prepared by dissolving 1 mg of the powder in 1 mL of ultrapure water and stored in the fridge at 4 °C. Test solutions for CE analysis were prepared daily from the stock solution at a concentration of 1 μg/mL (Hex-PAH) or 10 μg/mL (Hex-PAH:CD) in a sodium tetraborate buffer solution that was pH adjusted with 0.5 M boric acid. Samples were run immediately after preparation.

Complex Stability

The stability of the Hex-PAH:BCD-X12 complexes were studied at three Hex-PAH:BCD-X12 mole ratios (1:1, 1:2, and 1:5), with respect to the buffer concentration, pH, temperature, and agitation. To test the thermal stability, stock solutions were stored at 4 °C, room temperature (~22 °C), and 37 °C. Test solutions were prepared after 0.5 h, 1 h, 2 h, 4 h, 6 h, 24 h and 48 h of incubation, and analyzed immediately. The stability was estimated by comparing the area of the complex peak to the Hex-PAH peak, and calculating a stable complex ratio using the following equation:

$$A_{complex}/(A_{complex}+A_{Hex\text{-}PAH}) \quad (1)$$

where A is the peak area. To test the stability of the complex during agitation, the test solutions were stirred at varying arbitrary rates for 5 s on a vortex mixer (Vortex Genie 2, Fisher Scientific, Nepean, ON, Canada) immediately before CE separation.

RESULTS

The CE/LIF operating parameters were optimized with respect to pH (7.5 - 9), and buffer concentration, (10-80 mM sodium tetraborate) for the best complex stability and separation. Initially a 1:2 Hex-PAH:BCD-X12 mole ratio was used, based on the rationale that this ratio would be the most stable, based on the formation of a near neutral complex; Hex-PAH has a minus-12 charge whereas the BCD-X12 has a plus-7 charge. The 1:1 mole ratio was expected to have an excess of free Hex-PAH, whereas the 1:5 mole ratio and 1:2 mole ratio formulations were expected to have less free Hex-PAH and be more stable.

pH Optimization

Several buffers were initially investigated for their utility; however, only borate buffers afforded both

good separation and well defined peaks. The failure to detect complexes in phosphate buffers was attributed to the competition between the large excess of phosphate ions and the relatively small numbers of polynucleotide phosphate ions. Since a borate buffer was used, the pH study was limited to its buffering range (7.5-9.2). The pH of the medium is important since it will affect protonation of the charges on both components of the complex and consequently promote or disrupt the stability of the complex. In addition, if BCD-X12 is to be used as a vector for therapeutics, it must be stable at physiological conditions. Figure 2 shows the electropherograms of Hex-PAH:BCD-X12 complexes at several pH values and a constant sodium tetraborate buffer concentration of 10 mM. As sodium tetraborate solutions (10 mM) were adjusted to pH 9.0, 8.5, 8.0, and 7.5, using increasing volumes of 0.5 M boric acid, the resulting total borate concentration (TBC) changed to 10, 45, 100, and 160 mM, respectively. At pH 7.5, baseline separation was achieved. However, as the pH was increased (pH 8.5 and 9.0), the complex peak had completely disappeared. To ensure the decreasing stability of the complex was due to pH only and not the changing borate concentration of the buffer solution (ionic strength), additional tests were conducted using a constant ionic strength buffer containing 10 mM sodium tetraborate that was pH adjusted with acetic acid. The results of these tests (data not shown) were nearly identical to previous results, indicating that the pH alone affected complex stability. Since a pH of 7.5 gave the best separation and the highest stable complex ratio, it was used for the remainder of the study.

Buffer Concentration Optimization

The buffer concentration was adjusted by varying the concentration of sodium tetraborate to 10, 20, 40, 60, and 80 mM and pH adjusting to 7.5 with 0.5M boric acid. As a result, the TBC changed to 160, 210, 280, 310 and 330 mM, respectively. Figure 3 shows the effect of the total borate concentration on the separation of the Hex-PAH:BCD-X12 complex. At the lowest borate concentration (160 mM), the peaks were baseline resolved, and the peak area of the complex was at its maximum. As the buffer concentration increased, the area under the complex peak slowly decreased with respect to the Hex-PAH peak, and baseline separation was no longer achieved. At the highest borate concentration (330 mM), the complex peak was not observed. A buffer concentration of 10 mM (160 mM TBC) afforded the best results, so it was used in subsequent experiments.

Physical and Thermal Stability of the Hex-PAH:BCD-X12 complex Mole Ratio Stability

Three Hex-PAH:BCD-X12 formulations with mole ratios of 1:1, 1:2 and 1:5 were prepared. These mole ratios represent the mixing conditions during the preparation of the Hex-PAH:BCD-X12 complex, and do not necessarily represent the actual binding stoichiometry. Figure 4 shows that each mole ratio resulted in both a complex peak and a Hex-PAH peak. Interestingly, only one complex peak was detected in all three formulations, and the migration time of the complex peak was the same at all mole ratios, suggesting that the complex was the same in all cases (e.g., only one binding stiochiometry was stable). Figure 4 shows that the

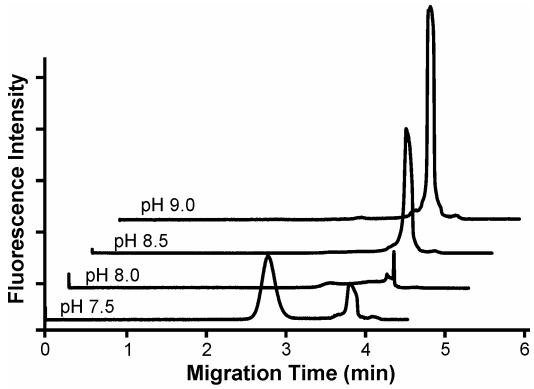

Figure 2: Effect of pH on the separation of the 1:2 Hex-PAH:BCD-X12 formulation. The electropherograms are offset in the x-axis for clarity. The first peak represents the Hex-PAH:BCD-X12 complex, and the second peak is the unbound Hex-PAH.

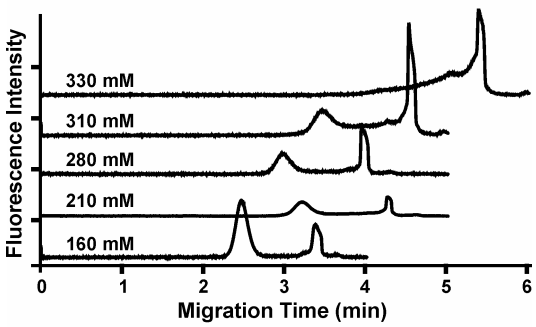

Figure 3: Influence of the total borate concentration (TBC) on the separation of the 1:2 Hex-PAH:BCD-X12 formulation. The first peak is the complex, and the second peak is the unbound Hex-PAH.

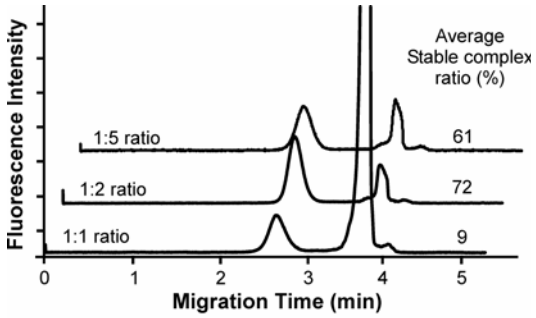

Figure 4: Separation of Hex-PAH:BCD-X12 formulations in which the molar ratios are 1:1, 1:2 and 1:5. The electropherograms are offset in the x-axis for clarity.

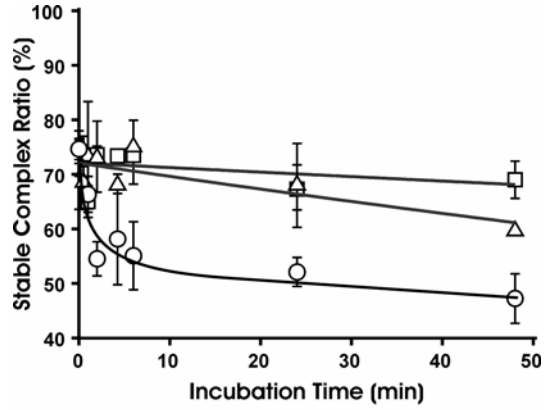

Figure 5: Stability of the 1:2 Hex-PAH:BCD-X12 formulation at 4 °C, 22 °C and 37 °C for 2 days. Square = 4 °C, Triangle = 22 °C, Circle = 37 °C

1:2 mole ratio was the most stable, having the highest stable complex ratio. The stability of the 1:5 formulation was nearly identical to that for the 1:2 formulation, whereas the 1:1 formulation had the lowest stable complex ratio. Since most of the work on the 1:2 formulation we were able to report a relative standard deviation (n=5) of 7.0 %. Assuming that the RSD is similar for the 1:5 formulation, we cannot state that the difference between the 1:2 and 1:5 formulations are statistically significant.

Temperature and Agitation

Figure 5 shows the thermal stability of the 1:2 Hex-PAH:BCD-X12 mole ratio formulation at three temperatures over a period of 2 days. No significant change was detected after storage at 4 °C for the duration of the study. At room temperature (nominally 22 °C), the complex continued to dissociate steadily over the duration of the study, and 37 °C, the complex initially quickly dissociated, and then continued to slowly dissociate

All samples were vortex mixed to ensure solution before analysis. Sample-to-sample variations of otherwise identical samples were occasionally observed; analysis of the experimental protocol identified vortex mixing intensity as the only uncontrolled variable in sample preparation protocol. Figure 6 shows the Hex-PAH:BCD-X12 stability at three arbitrary mixing intensities. Under these controlled conditions, the RSD values of the stable complex ratio using strong agitation conditions ranged from 10-13%, whereas, RSD values using gentle agitation were less than 5%. Gentle agitation also resulted in the highest stable complex ratio. It was therefore very important to only gently agitate the samples before analysis.

Hex-PAH binding to other CDs

CDs normally form inclusion complexes with appropriately sized lipophilic molecules. In the present work, it was postulated that 'charge-association' complexes are formed between the negatively-charged nucleic acid (Hex-PAH) and the positively-charged BCD-X12. To further clarify the nature of the association between these multiply-charged species, three other CDs were tested for their Hex-PAH binding ability: an unmodified BCD, a custom synthesized heptakis-(6-amino)-β-cyclodextrin (BCD-X4), and a custom synthesized heptakis-(6-azido-2-galactosyl)-β-cyclodextrin (BCD-X10). Figure 7 presents the results of the binding study using 1:2 formulations of these BCDs in a buffer containing 10 mM sodium tetraborate at pH 7.5. Of the four test CDs, only BCD-X12 formed a stable complex with the Hex-PAH under the optimized conditions, whereas BCD and BCD-X10 did not. This is confirmed by the migration time of the single peak, which corresponded to the migration of the Hex-PAH only. As expected on the basis of the 'charge-association' complexation model, BCD-X4 was able to complex Hex-PAH, but the broad peak in the electropherogram is indicative of complex instability under separation conditions.

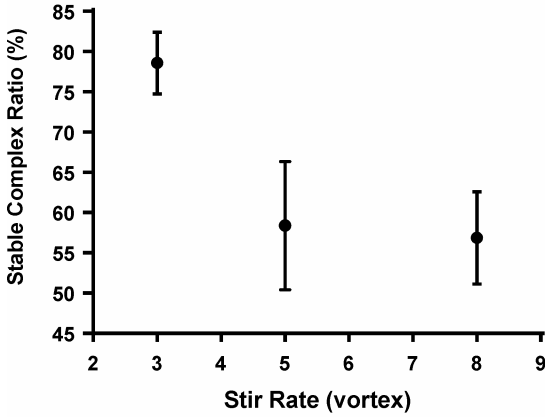

Figure 6: Effect of stir-rate on the stability of the 1:2 Hex-PAH:BCD-X12 formulation.

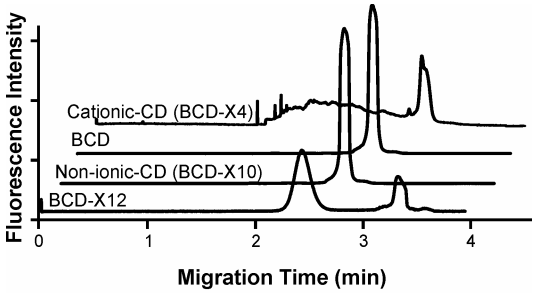

Figure 7: Electropherograms of the binding of the oligonucleotide to four different BCDs in 1:2 formulations: BCD-X12, BCD-X4 (both cationic), BCD-X10 and β-CD (non-cationic). The electropherograms are offset in the x-axis for clarity. The BCD-X12 trace shows complex and free Hex-PAH as sharp peaks, whereas BCD-X4 shows a broad complex 'hump' with a sharp Hex-PAH component, and the two non-ionic BCDs depict only free Hex-PAH.

DISCUSSION

The stability of the drug:vector complex is an important factor in drug delivery. The vector must be able to bind the therapeutic drug or gene strongly enough to deliver the drug to its target, while avoiding degradation by metabolic processes. However, it must also be weak enough to allow the drug to be released upon its arrival at the target. Factors that contribute to stability during administration include pH, drug:vector mole ratio, and temperature. Agitation conditions are also important to maintain complex stability during sample preparation.

Because of the buffer used in this study, pH values below 7.5 were not investigated. This pH; however, is very close to normal blood pH, which is about 7.4. At this pH, the complex was the most stable as shown by the presence of the large complex peak in Fig 3. As the pH was increased, the complex started to dissociate, and at pH values of 8.5 and 9, the complex peak had completely disappeared. Complex dissociation was likely due to the amine groups on the BCD-X12 being deprotonated, resulting in the BCD-X12 being less positively charged. Although the pKa of BCD-X12 was not determined, a good model compound is glucosamine, which has a pKa of 6.9. This would support the hypothesis that the complex is formed by electrostatic binding, and the loss of positive charge on the BCD-X12 would result in decreased binding of the negatively charged Hex-PAH oligomer.

One method to increase the apparent stability of the complex is to use a higher concentration of the vector than the drug. The rationale is that even though the complex dissociates during transport or storage, the massive excess of vectors in solution will bind to any free oligomer. The three mole ratios tested in this work revealed that even at high Hex-PAH:BCD-X12 mole ratios, the complex stability did not improve (Fig. 4). This was clearly observed by the presence of the large free oligomer peak in the electropherogram of the 1:5 formulation. This study also indicated that only one complex was formed at each of the three mole ratios, and that these complexes were electrophoretically identical to each other. The identity of the actual stoichiometry of the stable complex has not yet been determined.

Figure 6 shows the variation in the complex stability during various arbitrary mixing conditions. The plot clearly shows that gentle agitation (setting 3) resulted in a lower standard deviation in the stable complex ratio. This is an important consideration to follow, as it was found that strong and/or erratic mixing techniques seriously affected the apparent stability of the complex. Because of the relatively weak interactions between Hex-PAH and BCD-X12, strong mixing resulted in the complex falling apart in solution, and it was unable to re-associate with its binding partner.

Nucleic acid complexation could be a useful approach to the pharmaceutical development of orally available nucleic acid based therapeutics (34). The thermal stability of such complexes at storage and physiological temperatures is pragmatically important for both shelf-life and activity considerations. The study of thermal stability showed that higher temperatures resulted in greater dissociation (Fig. 5). This was not surprising because of the charge associated nature of the binding. The difference in the stable complex ratio; however, was only about 20 once it reached equilibrium after 2 hours of storage, indicating that the majority of the complexes (~70%) were still intact at physiological temperatures.

To show that the modified structure of BCD-X12 was required for complex formation, three other CDs were tested for their ability to complex with Hex-PAH. Two of the CDs were neutrally charged (BCD and BCD-X10) while the third, like BCD-X12, was cationically charged (BCD-X4) (Fig 1). Figure 7 shows that under the reported analytical conditions, only BCD-X12 was able to form stable complexes with Hex-PAH. Overall, this supports the concept of charge-association binding rather than inclusion complexation, since BCD-X10 and BCD are not cationically charged. The weak binding between BCD-X4 and Hex-PAH is perplexing, however, since both BCD-X12 and BCD-X4 are fully aminated (seven amino groups) at C-6. No clear explanation has been developed, but it may well relate to the large hydrophilic environment created by full galactosylation at C-2 of each BCD-X12 molecule. Inclusion complex formation with BCD-X12 may be difficult as the galactose and linker combined create a barrier approximately 9.8 Å deep, protecting the beta face of the CD and adenosine is only around 4.6 Å long. It would thus seem that the less hydrophilic adenosine moiety would be

effectively blocked from reaching the CD cavity for inclusion complex formation. Finally, since no stable complexes were formed with the neutral cyclodextrins (BCD-X10 and BCD), inclusion processes seem unlikely to play a large role in the binding of the Hex-PAH; however, they cannot be excluded on the basis of these studies.

CONCLUSIONS

A galactosylated-amino-β-cyclodextrin (BCD-X12) was successfully complexed with a 12-mer fluorescently-tagged arabinopolynucleotide (Hex-PAH) at 1:1, 1:2 and 1:5 cyclodextrin:polynucleotide mole ratios. Capillary electrophoresis indicated that only a single complex was formed between BCD-X12 and Hex-PAH at all formulation ratios, and that the complexes were electrophoretically identical to each other. Increasing the molar ratio beyond 1:2 did not contribute measurably to the stability of the complex. The stability of the Hex-PAH:BCD-X12 complex was shown depend both on storage temperature and stirring intensity; gentle agitation was crucial for run to run reproducibility. Of the four CDs tested, only BCD-X12 formed complexes with Hex-PAH that were stable under the reported analytical conditions. The binding stoichiometry and structure of the Hex-PAH:BCD-X12 complex are still under investigation. This initial study demonstrates that polycationic cyclodextrins have the potential to be developed as carriers of therapeutic nucleic acids.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support from the Natural Sciences and Engineering Research Council of Canada, Alberta Health and Wellness, the Canada Research Chairs Program (to XCL), and an Alberta Ingenuity Postdoctoral Fellowship (to JWG). This research was supported in part through a National Research Council IRAP grant to BioCyDex, Inc.

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